

## Antimicrobial Resistance and Virulence Genes of *Escherichia coli* Isolates from Swine in Ontario

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**A total of 318 *Escherichia coli* isolates obtained from diarrheic and healthy pigs in Ontario from 2001 to 2003 were examined for their susceptibility to 19 antimicrobial agents. They were tested by PCR for the presence of resistance genes for tetracycline, streptomycin, sulfonamides, and apramycin and of 12 common virulence genes of porcine *E. coli*. Antimicrobial resistance frequency among *E. coli* isolates from swine in Ontario was moderate in comparison with other countries and was higher in isolates from pigs with diarrhea than in isolates from healthy finisher pigs. Resistance profiles suggest that cephamycinases may be produced by  $\geq 8\%$  of enterotoxigenic *E. coli* (ETEC). Resistance to quinolones was detected only in enterotoxigenic *E. coli* ( $\leq 3\%$ ). The presence of *sul3* was demonstrated for the first time in Canada in porcine *E. coli* isolates. Associations were observed among *tetA*, *sul1*, *aadA*, and *aac(3)IV* and among *tetB*, *sul2*, and *strA/strB*, with a strong negative association between *tetA* and *tetB*. The *paa* and *sepA* genes were detected in 92% of porcine ETEC, and strong statistical associations due to colocation on a large plasmid were observed between *tetA*, *estA*, *paa*, and *sepA*. Due at least in part to gene linkages, the distribution of resistance genes was very different between ETEC isolates and other porcine *E. coli* isolates. This demonstrates that antimicrobial resistance epidemiology differs significantly between pathogenic and commensal *E. coli* isolates. These results may have important implications with regards to the spread and persistence of resistance and virulence genes in bacterial populations and to the prudent use of antimicrobial agents.**

Antimicrobial resistance (AMR) is recognized as a global problem in human and veterinary medicine. To estimate the extent of the AMR problem and to follow its evolution, surveillance programs have been established in many countries worldwide, including the National Antimicrobial Resistance Monitoring System (NARMS) in the United States (53) and the Canadian Integrated Program for Antimicrobial Resistance Surveillance in Canada (3). The majority of these programs are dedicated to the surveillance of AMR in agents of zoonoses and in indicator bacteria of the normal intestinal flora of animals (e.g., *Escherichia coli* and *Enterococcus* spp.). This represents an important first step in our efforts to understand and control AMR. Unfortunately, few surveillance programs include specific pathogens from animals, and most are dedicated to resistance phenotypes only. In numerous instances, several different genotypes can be at the origin of similar resistance phenotypes (14, 49, 50). Thus, assessing the diversity and distribution of resistance genes in bacterial populations represents a more detailed and potentially useful ad-

ditional tool for improving our understanding of AMR epidemiology (8).

In the case of *E. coli*, resistance to tetracyclines, sulfonamides, and streptomycin or spectinomycin is generally the most prevalent (3, 15, 21, 29; <http://www.arru.saa.ars.usda.gov/>). A number of recent studies have attempted to assess the distribution of the resistance genes for these major antimicrobial agents in *E. coli* populations of animal origin (5–7, 9, 10, 16, 21, 29, 35, 51, 52), but much remains to be done to draw valid comparisons between *E. coli* isolates from different animal populations. For instance, previous studies from our laboratory showed that the distribution of antimicrobial resistance genes from pathogenic *E. coli* isolates obtained from swine may differ significantly from those of other animal species (29). Many hypotheses can be invoked to explain such differences in the distribution of antimicrobial resistance genes in bacteria from different host populations. These include differences in antimicrobial use, the clonal nature of some pathogenic *E. coli* isolates, a lack of epidemiological and ecological links between *E. coli* isolates of different animal species, and sampling bias. AMR is also typically more frequent among pathogens than among commensal bacteria (15). This difference has generally been attributed to the more intense and repeated exposure of pathogens to antimicrobial agents. However, to the best of our knowledge, this hypothesis has never been formally tested and other factors may be at work. Physical linkages between antimicrobial resistance genes and specific virulence genes in pathogens may be another explanation (32). Such linkages of

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TABLE 1. Single PCR conditions and control strains<sup>e</sup>

Gene	Primer name	Primer sequence	Annealing (°C)	Fragment size (bp)	Positive control
<i>aadA</i>	4F <sup>a</sup>	GTGGATGGCGGCCTGAAGCC	68	525	AMR-002 <sup>d</sup>
	4R <sup>a</sup>	AATGCCAGTCGGCAGCG			
<i>strA</i>	2F <sup>a</sup>	CCTGGTGATAACGGCAATTC	55	546	AMR-009 <sup>d</sup>
	2R <sup>a</sup>	CCAATCGCAGATAGAAGGC			
<i>strB</i>	3F <sup>a</sup>	ATCGTCAAGGGATTGAAACC	55	509	AMR-009 <sup>d</sup>
	3R <sup>a</sup>	GGATCGTAGAACATATTGGC			
<i>tetA</i>	TetA-L <sup>b</sup>	GGCGGTCTTCTTCATCATGC	64	502	RO8 <sup>d</sup>
	TetA-R <sup>b</sup>	CGGCAGGCAGAGCAAGTAGA			
<i>tetB</i>	TetB-L <sup>b</sup>	CATTAATAGGCGCATCGCTG	64	930	PB#11 <sup>d</sup>
	TetB-R <sup>b</sup>	TGAAGGTCATCGATAGCAGG			
<i>tetC</i>	TetC-L <sup>b</sup>	GCTGTAGGCATAGGCTTGGT	64	888	PB#02 <sup>d</sup>
	TetC-R <sup>b</sup>	GCCGGAAGCGAGAAGAATCA			
<i>sul1</i>	Sul1-L <sup>b</sup>	GTGACGGTGTTCGGCATTCT	68	779	AMR-130 <sup>d</sup>
	Sul1-R <sup>b</sup>	TCCGAGAAGGTGATTGCGCT			
<i>sul2</i>	Sul2-L <sup>b</sup>	CGGCATCGTCAACATAACCT	66	721	AMR-130 <sup>d</sup>
	Sul2-R <sup>b</sup>	TGTGCGGATGAAGTCAGCTC			
<i>sul3</i>	Sul3-F <sup>c</sup>	GAGCAAGATTTTGTGAATCG	51	880	RL0044 <sup>c</sup>
	Sul3-R <sup>c</sup>	CATCTGCAGCTAACCTAGGGCTTTGGA			
<i>aac(3)IV</i>	Aac4-L <sup>d</sup>	TGCTGGTCCACAGCTCCTTC	59	653	AMR-075 <sup>d</sup>
	Aac4-R <sup>d</sup>	CGGATGCAGGAAGATCAA			

<sup>a</sup> Reference 31.<sup>b</sup> Reference 29.<sup>c</sup> Reference 44.<sup>d</sup> Data are from the present study.<sup>e</sup> All PCRs were done with the following temperature cycling: 1 cycle of 4 min at 95°C; 35 cycles, each consisting of 1 min at 95°C, 1 min at annealing temperature, and 1 min at 72°C; and 1 cycle of 7 min at 72°C.

genes on large transferable plasmids have been described sporadically in the past for enterotoxigenic *E. coli* (ETEC) isolates from swine and calves (19, 23, 25, 36) and for avian *E. coli* isolates (28). Nothing is known about the frequency of these associations among field isolates.

The first objective of this study was to obtain an estimate of frequency of resistance to common antimicrobial agents in ETEC isolates obtained from pigs in Ontario by an internationally standardized method. The second objective was to assess the diversity and distribution of the major resistance genes to tetracyclines, sulfonamides, streptomycin or spectinomycin, and apramycin in these ETEC isolates in other *E. coli* isolates from diarrheic pigs and in commensal *E. coli* isolates from swine of the same region. The third objective was to assess the distribution of the major virulence genes in these isolates and identify any associations between virulence and resistance genes in *E. coli* from this swine population.

#### MATERIALS AND METHODS

**Bacterial isolates.** One hundred and fifty *E. coli* isolates were systematically collected from cases of diarrhea in pigs submitted to the Animal Health Laboratory, University of Guelph, by veterinary practitioners and farmers from Ontario between January and October 2003. The *E. coli* isolates were isolated and identified, following standard procedures (45). Only *E. coli* isolates that agglutinated in a polyclonal anti-F4 antiserum or a polyvalent antiserum pool against the OK serogroups O138:K81, O139:K82, O141:K85ab, O141:K85ac, O45ac:K“E65,” O157:K“V17,” O115:K“V165,” O8:K“X105,” O7:K48, and O149:K91 (*Escherichia coli* Laboratory, Faculté de Médecine Vétérinaire, University of Montreal, Saint-Hyacinthe, Canada) were included in this collection. One isolate was collected per animal or group of animals submitted for analysis at each date, except when isolates presented clearly different phenotypic characteristics in hemolysis or agglutination. Ninety-five of these isolates originated from pigs on 67 farms in Ontario. Information on the farm of origin of the pigs from which the remaining 55 isolates were obtained was missing. The first isolate obtained from each of the 67 farms was used for later analysis of epidemiologically unrelated isolates. The approximate age of 101 of the pigs with diarrhea was known and

ranged from 1 to 16 weeks (median of 4 weeks). All the isolates from cases of diarrhea were serotyped at the Laboratory for Food-Borne Zoonoses, Public Health Agency of Canada, Guelph, Canada, by standard protocols (43). Thirty-five additional ETEC isolates recovered from pigs with diarrhea in Ontario between 1974 and 1987 (42) were used as a comparison to recent isolates for the detection of the sulfonamide resistance gene *sul3*.

A systematic random sample of 168 commensal *E. coli* isolates previously obtained from feces of healthy finisher pigs in 97 farms in Ontario between 2001 and 2002 were used for comparison to isolates from cases of diarrhea. The first isolate obtained from each of the 97 farms was used for analysis of epidemiologically unrelated isolates.

Control strains used for PCR are listed in Table 1. All the isolates used for the study were kept frozen at -70°C in brain heart infusion broth (Becton Dickinson, Sparks, MD) containing 20% glycerol until they were used.

**Antimicrobial susceptibility testing.** All the strains were tested for susceptibility to the following antimicrobial agents by the microdilution method recommended by NARMS (breakpoints are indicated in parentheses): ampicillin (≥32 µg/ml), amoxicillin-clavulanic acid (≥32 and ≥16 µg/ml, respectively), cefoxitin (≥32 µg/ml), ceftriaxone (≥64 µg/ml), ceftiofur (≥8 µg/ml), cephalothin (≥32 µg/ml), streptomycin (≥64 µg/ml), kanamycin (≥64 µg/ml), gentamicin (≥16 µg/ml), amikacin (≥64 µg/ml), tetracycline (≥16 µg/ml), chloramphenicol (≥32 µg/ml), sulfamethoxazole (≥512 µg/ml), trimethoprim-sulfamethoxazole (≥4 and ≥76 µg/ml, respectively), nalidixic acid (≥32 µg/ml), and ciprofloxacin (≥4 µg/ml). The strains from cases of diarrhea were additionally tested for resistance to trimethoprim, spectinomycin, and apramycin by disk diffusion and following the CLSI (formerly NCCLS) guidelines (38). The CLSI interpretation criteria for *E. coli* and *Pasteurellaceae* were used for trimethoprim and spectinomycin, respectively (39, 40). Based on the clear-cut bimodal distribution of inhibition zone diameters with apramycin, isolates were classified as resistant when the zone diameter was ≤10 mm.

**Detection of resistance and virulence genes.** The major resistance genes for tetracycline (*tetA*, *tetB*, and *tetC*), sulfonamides (*sul1*, *sul2*, and *sul3*), streptomycin-spectinomycin (*strA/strB*, *aadA*), and apramycin [*aac(3)IV*] were detected by PCR using the primers and protocols described in Table 1. The *aac(3)IV* PCR was validated by confirming the results obtained with 100 isolates from cases of diarrhea by dot blot hybridization (data not shown). The virulence genes for LT (*elt*), STa (*estA*), STb (*estB*), and F4 (*faeG*) on the one hand and F5 (*fanA*), F6 (*fasA*), F18 (*fedA*), and Stx2e (*stx2e*) on the other hand were detected in two separate multiplex PCRs using the Multiplex PCR kit from QIAGEN (Hilden, Germany). The cycling temperatures and primers used for these two multiplex

TABLE 2. Multiplex PCR conditions and control strains<sup>d</sup>

PCR	Gene	Primer name	Primer sequence	Annealing (°C)	Fragment size (bp)	Positive control
1	<i>estB</i>	STb-L <sup>a</sup>	TGCCTATGCATCTACACAAT	55	113	RO8 <sup>c</sup>
1		STb-R <sup>a</sup>	CTCCAGCAGTACCATCTCTA			
1	<i>estA</i>	Sta-L <sup>a</sup>	CAACTGAATCAGTTGACTCTT	55	158	RO8 <sup>c</sup>
1		Sta-R <sup>a</sup>	TTAATAACATCCAGCACAGG			
1	<i>elt</i>	LT-L <sup>a</sup>	GGCGTTACTATCCTCTCTAT	55	272	RO8 <sup>c</sup>
1		LT-R <sup>a</sup>	TGGTCTCGGTCAGATATGT			
1	<i>faeG</i>	F4-L <sup>a</sup>	GAATCTGTCCGAGAATATCA	55	499	RO8 <sup>c</sup>
1		F4-R <sup>a</sup>	GTTGGTACAGGTCTTAATGG			
2	<i>fanA</i>	F5-L <sup>a</sup>	AATACTTGTTTCAGGGAGAAA	55	230	B44 <sup>a</sup>
2		F5-R <sup>a</sup>	AACTTTGTGGTTAACTTCCT			
2	<i>fedA</i>	F18-L <sup>a</sup>	TGGTAACGTATCAGCAACTA	55	313	F107 <sup>a</sup>
2		F18-R <sup>a</sup>	ACTTACAGTGCTATTCGACG			
2	<i>fasA</i>	F6-L <sup>a</sup>	GTAACCTCCACCGTTTGTATC	55	409	P16M <sup>a</sup>
2		F6-R <sup>a</sup>	AAGTTACTGCCAGTCTATGC			
2	<i>Stx2e</i>	Stx2e-L <sup>a</sup>	AATAGTATACGGACAGCGAT	55	733	AMR-472 <sup>c</sup>
2		Stx2e-R <sup>a</sup>	TCTGACATTCTGGTTGACGC			
3	<i>astA</i>	EAST1-F <sup>b</sup>	TCGGATGCCATCAACACAGT	62	125	JG280 <sup>c</sup>
3		EAST1-R <sup>b</sup>	GTCGCGAGTGACGGCTTTGTAAG			
3	<i>paa</i>	PAACONS-L	GGCCCGCATACAGCCTTG	62	282	JG280 <sup>c</sup>
3		PAACONS-R	TCTGGTCAGGTCGTCATACTC			
3	<i>aidA-I</i>	AIDA-F <sup>b</sup>	ACAGTATCATATGGAGCCA	62	585	PD20 <sup>c</sup>
3		AIDA-R <sup>b</sup>	TGTGCGCCAGAATACTTA			
3	<i>sepA</i>	SEPA-B-L	TAAAACCCGCCGCCTGAGTA	62	611	JG280 <sup>c</sup>
3		SEPA-B-R	TGCCGGTGAACAGGAGGTTT			

<sup>a</sup> Bosworth and Casey, Abstr. 97th Gen. Meet. Am. Soc. Microbiol. 1997.<sup>b</sup> Reference 41.<sup>c</sup> Data are from the present study.<sup>d</sup> The multiplex PCR 1 and 2 were done with the following cycling: 1 cycle 15 min at 95°C; 30 cycles, each consisting of 1 min at 95°C, 1 min (plus 3 s at each cycle) at 55°C, and 2 min at 72°C; and 1 cycle for 10 min at 72°C. Multiplex PCR 3 was done with the following cycling: 1 cycle for 15 min at 94°C; 35 cycles, each consisting of 1 min at 94°C, 90 s at 62°C, and 1 min at 72°C; and 1 cycle for 10 min at 72°C.

PCRs are listed in Table 2 and have been previously validated (B. T. Bosworth and T. A. Casey, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. B-509, 1997). The *aidA*, *paa*, *astA*, and *sepA* genes were detected using the same Multiplex PCR kit and a third multiplex PCR developed for the present study (Table 2). The primers for *aidA* and *astA* were the same as those previously described by Ngeleka and collaborators (41). The primers for *paa* and *sepA* were chosen from conserved regions based on sequence alignment of these genes and their variants (*paa-I*, accession number AY547306; *sepA-I*, accession number AY604009) available from GenBank. Bacterial lysates used as templates for the PCRs were prepared as follows. A loopful of bacteria from a fresh overnight culture on a blood agar plate was resuspended homogeneously in 500 µl of water and heated at 95°C for 15 min. After being cooled to room temperature, the suspension was centrifuged for 3 min at maximum speed in a microcentrifuge. A 1-µl volume of the supernatant was used as a template for each 25-µl PCR mixture.

**Statistical analysis.** For the purpose of statistical analysis, isolates with intermediate susceptibility were classified, together with resistant isolates, as having reduced susceptibility. Confidence intervals for proportions were obtained using the exact binomial distributions option of NCSS (NCSS Statistical Software, Kaysville, Utah). Chi-square and Fisher's exact tests were performed for the analysis of associations using Statistix 7.0 for Windows (Analytical Software, Tallahassee, FL). Associations among genes were considered significant when *P* values were <0.05, in which case odds ratios and their 95% confidence intervals were calculated using the same software.

## RESULTS

**Antimicrobial susceptibility.** Based on genotyping, the 150 isolates from cases of diarrhea were classified for this analysis into 83 ETEC isolates (isolates positive for at least one enterotoxin gene and *faeG* or *fedA*) and 67 non-ETEC isolates (isolates that lacked a combination of enterotoxin and fimbrial genes). The results of the susceptibility testing for the whole set of isolates as well as for the subsets of epidemiologically unrelated isolates are reported in Table 3. There were no

isolates with reduced susceptibility to amikacin or ciprofloxacin, and only 1 isolate was resistant to nalidixic acid among the 318 isolates examined. The frequency of resistance to all the antimicrobial agents, except streptomycin, was consistently higher among isolates from cases of diarrhea than among those from healthy finisher pigs. Except for β-lactams and streptomycin, the frequency of resistance was also higher in ETEC isolates than in non-ETEC isolates from cases of diarrhea. Nineteen isolates from pigs from at least nine different farms presented a reduced susceptibility to ceftiofur (5 isolates, 13 isolates, and 1 isolate with MICs of >8 µg/ml, 8 µg/ml, and 4 µg/ml, respectively). Among these, 11 isolates from pigs from at least four different farms also had a reduced susceptibility to ceftriaxone (10 with a MIC of 16 µg/ml and 1 with a MIC of 32 µg/ml). These isolates with reduced susceptibility to extended-spectrum cephalosporins were all resistant to ceftiofur (MIC ≥ 16 µg/ml) and to amoxicillin-clavulanic acid, thus presenting a resistance profile compatible with the presence of a cephamycinase. Reduced susceptibility to ceftiofur was statistically associated with resistance to gentamicin (*P* = 0.036) and chloramphenicol (*P* = 0.007). Reduced susceptibility to ceftriaxone was also associated with resistance to gentamicin (*P* = 0.002), chloramphenicol (*P* = 0.011), and (additionally) kanamycin (*P* = 0.030). The number of epidemiologically unrelated isolates with reduced susceptibility to extended-spectrum cephalosporins was too low to provide accurate estimates of odds ratios for these associations.

**Distribution of antimicrobial resistance genes.** The frequencies of the major resistance genes for tetracycline, streptomycin, sulfonamides, and apramycin are reported in Table 4. All

TABLE 3. Frequency of resistance to antimicrobial agents among *E. coli* isolates from pigs with diarrhea and healthy finisher pigs<sup>a</sup>

Antimicrobial agent	Isolate parameter (total no. of isolates/no. epidemiologically unrelated)		
	% ETECs ( <i>n</i> = 83/36)	% Non-ETEC ( <i>n</i> = 67/33)	% Healthy finisher pig isolates ( <i>n</i> = 168/97)
Ampicillin	57/56 (38–72)	69/73 (54–87)	29/29 (20–39)
Amoxicillin-clavulanic acid	19/17 (6–33)	18/21 (9–39)	0/0 (0–4)
Cephalothin	63/67 (49–81)	48/58 (39–75)	4/2 (0–7)
Cefoxitin	19/19 (8–36)	15/18 (7–36)	0/0 (0–4)
Ceftiofur	13/11 (3–26)	12/15 (5–32)	0/0 (0–4)
Ceftriaxone	10/8 (2–22)	4/3 (0–16)	0/0 (0–4)
Streptomycin	42/44 (28–62)	63/46 (28–64)	51/49 (38–59)
Spectinomycin <sup>b</sup>	87/83 (67–94)	45/52 (34–69)	ND
Kanamycin	64/56 (38–72)	30/15 (5–32)	10/10 (5–18)
Gentamicin	23/28 (14–45)	15/12 (3–28)	2/1 (0–6)
Apramycin <sup>b</sup>	17/17 (6–33)	9/6 (1–20)	ND
Amikacin	0/0 (0–10)	0/0 (0–11)	0/0 (0–4)
Sulfamethoxazole	93/89 (74–97)	79/73 (54–87)	60/61 (50–71)
Trimethoprim <sup>b</sup>	28/35 (20–53)	32/28 (14–47)	ND
Trimethoprim-sulfonamide	30/39 (23–57)	34/24 (11–42)	10/9 (4–17)
Nalidixic acid	1/3 (0–14)	0/0 (0–11)	0/0 (0–4)
Ciprofloxacin	0/0 (0–10)	0/0 (0–11)	0/0 (0–4)
Tetracycline	96/100 (90–100)	94/91 (76–98)	79/81 (72–89)
Chloramphenicol	70/61 (43–77)	37/45 (28–64)	24/19 (11–28)

<sup>a</sup> The first number for each antimicrobial agent and category of isolates represents overall results, whereas the second number and the numbers in brackets present results from epidemiologically unrelated isolates only and the corresponding 95% confidence interval on this estimate, respectively. ND, not determined.

<sup>b</sup> Disk diffusion.

35 ETEC isolates recovered between 1974 and 1987 were *sul3* negative (other resistance genes were not tested for these isolates). The correlation between genotype (absence or presence of a major resistance gene) and phenotype (susceptibility or reduced susceptibility) was high for tetracycline (98% agreement), sulfonamides (98% agreement), and apramycin (100% agreement for isolates from cases of diarrhea). The correlation between resistance to gentamicin and the presence of the *aac(3)IV* gene was also high (96%) with 11 of 12 disagreements attributable to the likely presence of a gentamicin resistance gene other than *aac(3)IV*. In contrast with the other resistances, the agreement between genotypes and phenotypes for streptomycin was poor (66% agreement). In the majority of cases, this disagreement was due to the presence of an *aadA* gene in isolates classified as susceptible to streptomycin (MIC  $\geq 64$   $\mu$ g/ml). This later result was confirmed repeatedly with freshly made lysates. Several of these PCR products from

streptomycin-susceptible isolates were examined by DNA sequencing and confirmed to be all *aadA* (data not shown). The agreement between the *aadA* genotype and the spectinomycin susceptibility phenotype (89% agreement) on one side and between the *strA-strB* phenotype and the streptomycin phenotype among *aadA*-negative isolates (88%) was better. In the case of spectinomycin, 14 of the 16 disagreements were due to the presence of an *aadA* gene in isolates classified as susceptible. In the case of streptomycin among *aadA*-negative isolates, 8 of 13 disagreements were associated with the absence of *strA-strB* in resistant isolates.

The simultaneous presence of more than one gene conferring resistance to the same antimicrobial agent was rarely observed for tetracycline (4% of 318 isolates) but more frequently for sulfonamides (15%) and streptomycin (34% with 15% due to the combination *strA* plus *strB* alone). Associations between major resistance genes are reported in Table 5. Ex-

TABLE 4. Distribution of major resistance genes for tetracycline, sulfonamides, streptomycin, and apramycin<sup>a</sup>

Resistance gene	Isolate parameter (total no. of isolates/no. epidemiologically unrelated)		
	% ETEC ( <i>n</i> = 83/36)	% Non-ETEC ( <i>n</i> = 67/33)	% Healthy finisher pig isolates ( <i>n</i> = 168/97)
<i>tetA</i>	89/92 (77–98)	37/36 (20–55)	32/33 (24–43)
<i>tetB</i>	12/11 (3–26)	63/58 (39–74)	46/50 (39–60)
<i>tetC</i>	1/0 (0–10)	0/0 (0–11)	5/3 (1–9)
<i>sul1</i>	72/69 (52–84)	33/30 (16–49)	21/19 (11–28)
<i>sul2</i>	31/36 (21–54)	42/36 (20–55)	24/28 (19–38)
<i>sul3</i>	15/11 (3–26)	24/27 (13–46)	26/24 (16–33)
<i>aadA</i>	89/86 (71–95)	60/70 (51–84)	55/56 (45–66)
<i>strA</i>	31/31 (16–48)	49/36 (20–55)	28/24 (16–33)
<i>strB</i>	34/33 (19–51)	51/39 (23–58)	28/24 (16–33)
<i>aac(3)IV</i>	18/17 (6–33)	9/6 (1–20)	1/0 (0–4)

<sup>a</sup> The first number for each gene and category of isolates represents overall results, whereas the second number and the numbers in brackets present results from epidemiologically unrelated isolates only and the corresponding 95% confidence interval on this estimate, respectively.

TABLE 5. Pairwise statistical associations between major antimicrobial resistance genes<sup>a</sup>

	<i>tetA</i>	<i>tetB</i>	<i>sul1</i>	<i>sul2</i>	<i>sul3</i>	<i>aadA</i>	<i>strA</i>	<i>strB</i>	<i>aac(3)IV</i>
<i>tetA</i>									
<i>tetB</i>	0.02 (0.01–0.07)		10.30 (3.89–27.27)	–	–	2.43 (1.11–5.33)	–	–	–
<i>sul1</i>	9.99 (4.50–22.17)	0.27 (0.13–0.56)	0.29 (0.12–0.72)	–	–	28.08 (3.68–214.15)	4.64 (1.91–11.28)	4.93 (2.03–11.98)	–
<i>sul2</i>	–	–	0.46 (0.21–0.99)	0.32 (0.10–0.99)	0.18 (0.04–0.83)	–	–	0.36 (0.12–1.14)	–
<i>sul3</i>	–	–	0.28 (0.10–0.76)	–	–	–	9.46 (3.92–22.84)	8.63 (3.62–20.58)	–
<i>aadA</i>	3.01 (1.53–5.96)	–	52.93 (7.07–396.14)	–	–	2.67 (1.09–6.56)	–	0.37 (0.17–0.81)	–
<i>strA</i>	–	2.45 (1.23–4.92)	–	13.49 (6.02–30.26)	–	–	–	N/A	–
<i>strB</i>	–	2.44 (1.23–4.85)	–	13.49 (6.07–30.01)	–	0.46 (0.23–0.91)	N/A	–	–
<i>aac(3)IV</i>	8.8 (1.06–73.22)	–	7.09 (1.38–36.39)	–	–	–	–	–	–

<sup>a</sup> The numbers represent odds ratios for the associations between resistance genes (95% confidence intervals are in parenthesis). –, no statistically significant association detected. The numbers below the diagonal represent values obtained with all the epidemiologically unrelated isolates ( $n = 166$ ), and numbers above the diagonal represent those obtained after exclusion of ETEC isolates ( $n = 130$ ). N/A, statistically highly significant positive associations but calculation of odds ratios not applicable.

cept for apramycin, for which the number of resistant isolates from healthy pigs was too low, significant associations observed for the total population of epidemiologically unrelated isolates were confirmed after exclusion of the ETEC strains.

**Distribution of virulence genes.** The frequencies of the major virulence genes are reported in Table 6. A total of 152 isolates were negative for all the virulence genes under investigation (66% and 27% of the isolates from healthy finisher pigs and from pigs with diarrhea, respectively). The following serotypes were observed among those from pigs with diarrhea and presenting none of the virulence genes under investigation: O2:NM, O4:H5, O4:H27, O8:H9, O9:NM, O15:H45, O17:H10, O17:NM, O19:H10, O19:NM, O20:NM, O21:H32, O39:H48, O64:H25, O68:H30, O68:H?, O69:H?, O74:H42, O79:NM, O83:H31, O88:H11, O88:H8, O106:H?, O107:H27, O112ab:H8, O139:NM, O143:H4, O153:NM, O170:H28, O?:H10, O?:H19, and O?:NM. Isolates with the *elt*, *estB*, and *faeG* combination of virulence genes typical for porcine ETEC (83 isolates) all belonged to serogroups O149, O157, and O8. All ETEC isolates that also carried the *estA* gene belonged to serogroup O149 and were positive for both *paa* and *sepA* (69 isolates). The *paa* gene was seen occasionally in non-ETEC isolates, either in combination with *aidA* (one isolate), *astA* (one isolate), or *fedA* and *astA* (one isolate). The *sepA* gene was also seen in non-ETEC isolates, either alone (three isolates), or in combination with *estA* (four isolates). The *aidA* gene was detected in non-ETEC isolates alone (three isolates) or in combination with *paa* (one isolate), *fedA* (two isolates), or *fedA* and *faeG* (one isolate). Those isolates from diarrheic pigs and positive for *aidA* belonged to the serogroups O139 (three isolates), O138 (one isolate), O45 (one isolate); one belonged to a nonidentifiable O serogroup. Statistically significant associations between virulence genes and resistance genes are presented in Table 7.

## DISCUSSION

**Resistance phenotypes.** With the notable exception of quinolones, the results from this study show alarming resistance frequencies in *E. coli* from swine in Ontario. This was particularly the case for tetracyclines, sulfonamides, spectinomycin, ampicillin, and cephalothin. In agreement with a previous report (29), the distribution of inhibition zone diameters for spectinomycin and the correlation with genotypes show that a breakpoint between 16 mm and 18 mm would be more appropriate for *E. coli* than the one between 13 mm and 14 mm for *Pasteurellaceae* used here. Consequently, the true prevalence of spectinomycin resistance among *E. coli* isolates from cases of diarrhea is approximately 20% higher than the one presented in Table 3. Many *aadA*-positive isolates with reduced susceptibility to spectinomycin have MICs for streptomycin that are <64 µg/ml. This suggests that the NARMS microdilutions for streptomycin may have to be extended toward concentrations of <32 µg/ml and that the NARMS and Canadian Integrated Program for Antimicrobial Resistance Surveillance networks may possibly fail to detect a significant number of *aadA*-positive isolates. This also supports the low cut point (32 µg/ml) chosen for streptomycin MIC interpretation by surveillance programs such as the Danish monitoring program DANMAP (15). Thus, the true prevalence of streptomycin resis-

TABLE 6. Distribution of major virulence genes<sup>a</sup>

Virulence gene	Isolate parameter (total no. of isolates/no. epidemiologically unrelated)		
	% ETEC ( <i>n</i> = 83/36)	% Non-ETEC ( <i>n</i> = 67/33)	% Healthy finisher pig isolates ( <i>n</i> = 168/97)
<i>elt</i>	100/100 (90–100)	0/0 (0–11)	0/0 (0–4)
<i>estA</i>	83/86 (71–95)	1/0 (0–11)	2/2 (0–7)
<i>estB</i>	100/100 (90–100)	1/0 (0–11)	3/1 (0–6)
<i>astA</i>	99/100 (90–100)	27/33 (48–82)	30/29 (20–39)
<i>stx2e</i>	0/0 (0–10)	0/0 (0–11)	2/2 (0–7)
<i>faeG</i>	100/100 (90–100)	5/6 (1–20)	0/0 (0–4)
<i>fanA</i>	1/3 (0–14)	0/0 (0–11)	0/0 (0–4)
<i>fasA</i>	0/0 (0–10)	0/0 (0–11)	0/0 (0–4)
<i>fedA</i>	1/0 (0–10)	6/12 (3–28)	1/1 (0–6)
<i>aidA</i>	0/0 (0–10)	7/12 (3–28)	1/0 (0–4)
<i>paa</i>	88/92 (78–98)	3/0 (0–11)	1/2 (0–7)
<i>sepA</i>	93/92 (78–98)	7/6 (1–20)	2/1 (0–6)

<sup>a</sup> The first number for each gene and category of isolates represents overall results, whereas the second number and the numbers in brackets present results from epidemiologically unrelated isolates only and the corresponding 95% confidence interval on this estimate, respectively.

tance in porcine *E. coli* isolates obtained in Ontario is also likely to be much higher than the estimate presented in Table 3.

Except for ceftiofur, the resistance frequencies observed in ETEC isolates and cases of diarrhea in general were similar to those described in recent publications from Canada (2, 24, 35, 42). The frequency of ceftiofur resistance (11 to 13%) was twice to three times as high as in these previous studies. This is consistent with a possible increase in resistance to extended-spectrum cephalosporins in porcine ETEC isolates and suggests that these may represent an important reservoir of resistance genes of public health significance (54). Canadian porcine ETEC isolates present, for most antimicrobial agents tested, an intermediate position between those isolates from countries with low resistance rates such as Switzerland and Denmark (15, 29) and those with very high resistance rates such as Spain and Korea (13, 33). The only clear exception to this was resistance to quinolones (nalidixic acid and ciprofloxacin), which is less frequent in Canadian porcine isolates than those from other countries. Since fluoroquinolones are not registered for use in pigs in Canada, this does not come as a surprise.

As expected, clinical isolates (cases of diarrhea) were more frequently resistant than isolates from healthy animals for the majority of the antimicrobial agents examined. Previous studies have shown that resistance frequencies are usually higher in

younger animals (34). Since our isolates from healthy pigs were, on average, from older animals than the isolates from pigs with diarrhea, the observed difference could be in part due to this latter factor. Nevertheless, except for  $\beta$ -lactams, the association between pathogens and resistance was also apparent when comparing ETEC isolates with other non-ETEC isolates from cases of diarrhea without clear pathogenic potential and originating from the same source population.

**Resistance genotypes.** As in recent studies from other locations (10, 21, 29), resistance to tetracycline in porcine *E. coli* isolates from Ontario was mostly due to *tetA* and *tetB*. This observation is consistent with another Canadian study (35) and with a Norwegian study (51), showing that *tetA* and *tetB* were predominant among recent porcine ETEC isolates and porcine commensal *E. coli* isolates, respectively. All three known genes encoding resistance to sulfonamides were found in the population studied. These include the *sul3* gene recently described for *E. coli* and *Salmonella enterica* from different animal species and humans in Europe (4, 20–22, 44, 47). The *sul3* gene was recently detected for the first time in North America (7). We found this gene only in porcine *E. coli* isolates from 2001 to 2003 but not in porcine ETEC isolates from Ontario obtained between 1974 and 1987, thus clearly supporting the hypothesis made by several researchers that *sul3* has emerged only recently. Similarly to *tetA*, *sul1* and *aadA* were more frequent in ETEC isolates than in other porcine isolates. Since

TABLE 7. Pairwise statistical associations between major antimicrobial resistance genes and virulence genes<sup>a</sup>

	<i>tetA</i>	<i>tetB</i>	<i>sul1</i>	<i>sul2</i>	<i>sul3</i>	<i>aadA</i>	<i>strA</i>	<i>strB</i>	<i>aac(3)IV</i>
<i>elt</i>	21.50 (6.25–74.04)	0.12 (0.04–0.35)	8.28 (3.63–18.86)	–	–	4.27 (1.56–11.69)	–	–	12.80 (2.46–66.58)
<i>estA</i>	62.58 (8.28–472.93)	0.06 (0.01–0.26)	5.52 (2.45–12.46)	–	–	–	–	–	14.56 (2.79–76.02)
<i>estB</i>	22.67 (6.59–78.01)	0.11 (0.04–0.33)	8.93 (3.92–20.33)	–	–	4.46 (1.63–12.20)	–	–	12.29 (2.37–63.86)
<i>astA</i>	2.49 (1.33–4.67)	–	2.79 (1.42–5.47)	–	–	–	–	–	9.26 (1.11–77.10)
<i>faeG</i>	16.80 (5.60–50.43)	0.14 (0.05–0.39)	6.87 (3.12–15.14)	–	–	4.66 (1.71–12.73)	–	–	28.68 (3.40–241.75)
<i>fedA</i>	–	–	–	–	–	–	–	–	17.22 (2.41–123.04)
<i>aidA</i>	–	NA	–	–	–	–	–	–	–
<i>paa</i>	32.63 (7.48–142.27)	0.09 (0.03–0.30)	6.45 (2.88–14.48)	–	–	3.18 (1.24–8.19)	–	–	13.35 (2.56–69.50)
<i>sepA</i>	11.32 (4.12–31.11)	0.20 (0.08–0.51)	6.97 (3.10–15.61)	–	–	4.27 (1.56–11.69)	–	–	12.80 (2.46–66.58)

<sup>a</sup> The numbers represent odds ratios for the associations between genes (95% confidence intervals in parenthesis). –, no statistically significant association detected. Only epidemiologically unrelated isolates were used for this analysis (*n* = 166). NA, statistically highly significant positive associations but calculation of odds ratios not applicable (*P* = 0.032).

both *sul1* and *aadA* are usually parts of integrons (17, 18), our observations strongly suggest that integrons are more widespread among ETEC isolates than among other porcine *E. coli* strains. The apramycin resistance gene *aac(3)IV* also provides resistance to gentamicin and tobramycin; the origin of this resistance gene, sometimes found in bacteria isolated from humans, has been linked to animal sources (11, 26, 27, 48). Our results show that this gene is more frequent among ETEC isolates than among other porcine *E. coli* isolates in Ontario and are in agreement with observations based on phenotypes made in other countries (15). This suggests that ETEC may represent a significant reservoir for this gene of public health significance.

Numerous statistical associations were observed among resistance genes. Positive associations may be the consequence of the colocation of resistance genes on a single mobile genetic element such as a plasmid, a transposon, or an integron. Globally, two groups of associations seem to emerge from our results. An association between *tetA*, *sul1*, *aadA*, and *aac(3)IV* was present on one side; an association between *tetB*, *strA*, *strB*, and *sul2* appeared on the other side. The potential clonal nature of ETEC isolates (most of them belonged to the O149 serogroup and all were isolated during a 1-year period in a single province) could be a source of bias and could have suggested associations where there are none. However, most of these associations remained significant when excluding ETEC isolates from our analysis. This strongly supports the reliability of these findings. In addition, a biological basis for such associations is already known for the genes investigated. This includes, for instance, the association of *sul1* and *aadA* as parts of integrons, the association of *strA* and *strB* as a requirement for high-level expression of streptomycin resistance (12), and the association of these two latter genes with *sul2* on RSF1010 and other widespread plasmids (46). The relatively strong association between *tetA* and *sul1* (and to a lesser extent with *aadA*) suggests that *tetA*-carrying transposons such as Tn1721 (1) and other integron-carrying transposons (30) frequently coexist on composite mobile genetic elements in porcine *E. coli*. These mobile elements are likely to be large transferable plasmids. On the other hand, negative associations, such as the one between *tetA* and *tetB*, have already been observed by other researchers and are probably due to plasmid incompatibilities (35).

**Virulence genes.** As expected, virulence genes were more frequent in isolates from cases of diarrhea than in isolates from healthy animals. Among those isolates from diarrhea, the typical ETEC virulence genes *elt*, *estB*, and *faeG* were the most frequent. All the isolates with these virulence genes belonged to the classical ETEC serogroups O8, O157, and O149 (37). The high prevalence of *estA* among the serogroup O149 ETEC is in complete agreement with the emergence of a new *estA*-positive O149:H10 observed recently in pigs in Ontario (42). Similarly, the high prevalence of *paa* and *sepA* among ETEC isolates is also in agreement with the recent description of the new combined antimicrobial resistance and virulence pTENT2 plasmid in porcine ETEC (P. Boerlin and C. L. Gyles, Abstr. 104th Gen. Meet. Am. Soc. Microbiol., abstr. B-355, 2004). Consequently, the vast majority of recent porcine ETEC in Ontario not only possess the classical virulence genes of ETEC (*elt*, *estB*, *astA*, and *faeG*) and an additional *estA* gene, but also

carry two other potential virulence genes not known to have widely occurred previously in ETEC. Some of the isolates from cases of diarrhea lacking the classical ETEC virulence pattern carried virulence genes. Only a very few isolates from healthy finisher pigs did so too. This suggests that these virulence genes might have been implicated in the pathogenesis of the observed diarrhea.

**Association between virulence and antimicrobial resistance genes.** The consistently higher prevalence of AMR observed in ETEC was confirmed by the presence of associations between antimicrobial resistance and virulence genes. A more detailed analysis shows that the strongest associations were between *tetA* and *estA* and between *tetA* and *paa* (Table 7). This statistical association is easily explained by the clustering of *tetA*, *estA*, *paa-1*, and *sepA-1* on the pTENT2 plasmid of porcine ETEC from Ontario (Boerlin and Gyles, Abstr. 104th Gen. Meet. Am. Soc. Microbiol. 2004). The strongest negative associations found were between *tetB* and *estA* and between *tetB* and *paa*. Together with our observation that *tetA* and *tetB* were also negatively associated, this suggests that *tetB* in porcine *E. coli* isolates from Ontario is frequently located on plasmids of the same incompatibility group as pTENT2. The *aac(3)IV* gene also showed a strong association with ETEC and with *faeG* in particular. It is therefore likely that *aac(3)IV* is located on the same plasmid as *faeG* in some of our ETEC isolates and may become fixed in porcine *E. coli* populations in that way. Further investigations are warranted to clarify this point. The difference in prevalence of chloramphenicol and of kanamycin resistance between porcine *E. coli* from cases of diarrhea and healthy animals, as well as between ETEC and non-ETEC isolates from pigs with diarrhea, suggests that further linkages of resistance and virulence genes on plasmids are likely to be occurring; preliminary results in our laboratory confirm this hypothesis.

**Conclusion.** This study not only confirms that antimicrobial resistance is more frequent in pathogenic than in other porcine *E. coli* strains, but also shows that the resistance genes found in ETEC isolates are different from those of other porcine *E. coli* isolates and that clear associations exist between specific resistance and virulence genes. It shows that the *paa* and *sepA* virulence genes are widespread among porcine ETEC isolates and preliminary results in our laboratory confirm that not only these two genes but also plasmids related to pTENT2 are widespread in recent porcine ETEC isolates in Ontario. The confirmed and suspected links between resistance and virulence genes observed in the present study are worrisome in two ways. First, the use of antimicrobial agents may select for bacteria carrying virulence genes. This could accelerate the spread of virulence genes within bacterial populations and enhance the emergence of new pathogens or of pathogens with increased virulence potential, such as the newly emerged *estA*-, *paa*-, *sepA*-positive ETEC isolates in Ontario. It is conceivable that in some farm environments, the use of antimicrobials to treat diarrhea is in fact exacerbating the diarrhea problem and that routine in-feed use of antimicrobials may maintain bacterial strains harboring virulence genes. These possibilities have implications in the ongoing consideration of what constitutes prudent antimicrobial use in swine medicine and production. Second, resistance genes may be stabilized and fixed in pathogen populations by their linkage to virulence genes. Besides

the possible role of increased selective pressure by repeated exposure to therapeutic agents, this is a likely cofactor in the increased antimicrobial resistance frequency observed among pathogens.

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